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Rapid differentiation of archaebacteria from eubacteria by diffuse reflectance Fourier-transform IR spectroscopic analysis of lipid preparations

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Summary

A rapid method has been developed to distinguish pure cultures of archaebacteria from eubacteria. The strong acid methanolyzate of the total lipid extract was prepared by a simplified two-step process, and the diffuse reflectance Fourier-transform IR spectrum recorded. The height of the ester peak, expressed as a percent of the methyl peak, was chosen to distinguish the two kingdoms. The method was developed using four species of eubacteria and three species of archaebacteria, and then tested on another species of each. Minimum sample size for reliable results was 1 mg dry wt cells.

Key words: Archaebacterium; Biotechnology; Eubacterium; Fourier-transform IR spectroscopy; Lipid analysis

Introduction

Increasing interest in biotechnological applications of archaebacteria and their enzymes has led to the isolation of thousands of new strains from, for example, deep-sea hydrothermal vents. The number of isolates and archaebacterial species that do not fit into known groups, such as alkalophiles [1] and a sulfate reducer [2], suggest the need for rapid routine methods to accurately classify isolates as archaebacteria or eubacteria. The isoprenoid ether lipids of archaebacteria and the ester-linked long-chain fatty acids of eubacteria reliably distinguish these groups [3], with the exception of a eubacterium which contains both ester and ether linkages [4]. The extraction, fractionation, and derivatization of lipids, and their analysis by gas chromatography [5] and

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supercritical fluid chromatography [6] will reliably classify an isolate as either archaebacterial or eubacterial. However, the methods used for the detailed analysis of microbial lipids are not appropriate for a screening procedure. A rapid procedure has been reported utilizing thin-layer chromatography of whole cell methanolyzates [7]. The procedure required 100 mg dry wt of cells which may not be available or economically feasible.

The method presented in this report is reliable using as little as 1 mg dry wt cell material. The lipid extraction and degradation procedures were greatly simplified for this assay. For each sample of lyophilized cell material analysed, only three test tubes are used and 21 ml of reagents consumed. 20 samples were prepared in 1 work day. This compares with 10 samples prepared in 4 days for routine gas chromatographic analysis. Strong acid methanolysis of total lipid extracts was used to reduce the interfering peaks in the IR spectra. IR spectra were obtained by diffuse reflectance IR Fourier-transform (DRIFT). The ratio of the peak height of ester carbonyl stretch at 1743 cm^{-1} to the largest peak in the spectrum (the methyl stretch at 2924 cm^{-1}) was chosen to distinguish archaebacteria from eubacteria. The method reliably distinguished three species of archaebacteria and four of eubacteria. The sensitivity of the method was tested against an additional archaebacterial and eubacterial pair at a series of sample weights from 30 to $\approx$ 0.5 mg. The method reliably classified the samples with as little as 1 mg dry wt cell material.

Materials and Methods

Organisms

Lyophilized cells of *Thermodesulfotobacterium commune* [4], *Thermoplasma acidophilum* [8], and *Sulfolobus acidocaldarius* [9] were the generous gift of Thomas Langworthy. Live cultures of *Methanobacterium formicicum* and *Methanococcus maripaludis* were obtained from William Whitman and maintained by the methods of Balch [10] and Whitman [11], respectively. Lyophilized cells of *Escherichia coli, Clostridium welchii, Bacillus subtilis*, and *Micrococcus lysodeikticus* were purchased from Sigma Chemical, Saint Louis, Missouri.

Reagents

All glassware used was heated at 450 °C in a muffle furnace for 4 h. Plasticware was washed with chloroform/methanol and dried in a 100 °C oven. Chloroform, methanol, acetone, and hexane were nanopure grade from Burdick and Jackson. Hydrochloric acid was from Mallinkrodt. The water used was Barnstead nanopure stored over chloroform to extract residual lipids and prevent bacterial growth. Silicic acid was 200 mesh from Clarkson Chemical, Williamsport, Pennsylvania. Potassium bromide was IR grade from Sigma Chemical. Batyl alcohol (1-*o*-octadecyl-glycerol), 1-eicosanol, 1,2-di-*o*-hexadecyl-glycerol, phytol, and tripalmitin (Sigma Chemical) were used as IR standards.

Lipid preparation

Weighed samples of lyophilized cell material (0.3 – 30 mg) were placed in test tubes with Teflon-lined screw caps. For each group of samples, a blank test tube was carried

through all lipid procedures. A modification of the extraction method of Bligh and Dyer was used [12]. 2 ml of chloroform, 4 ml of methanol, and 1.8 ml of lipid-free water were added and the lipid extracted with sonication for 2 h. 2 ml each of chloroform and water were added, the samples were thoroughly mixed, and centrifuged. The lower organic phase was transferred to a clean test tube and the total lipid extract was recovered by removing the solvent with a stream of dry N_2 at 37 °C.

A strong acid methanolysis (SAM) of the total lipid extract was performed [13]. The total lipid was dissolved in 1 ml of methanol : chloroform : concentrated hydrochloric acid (10 : 1 : 1), and heated for 1 h at 100 °C To the cooled methanolyzate was added 2 ml each lipid-free water and hexane : chloroform (4 : 1). The mixture was thoroughly mixed, centrifuged, and the upper organic layer transferred. The methanolyzate was extracted twice more with 2 ml hexane chloroform and the solvent was removed from the pooled organic extracts in a stream of dry N_2 at 37 °C.

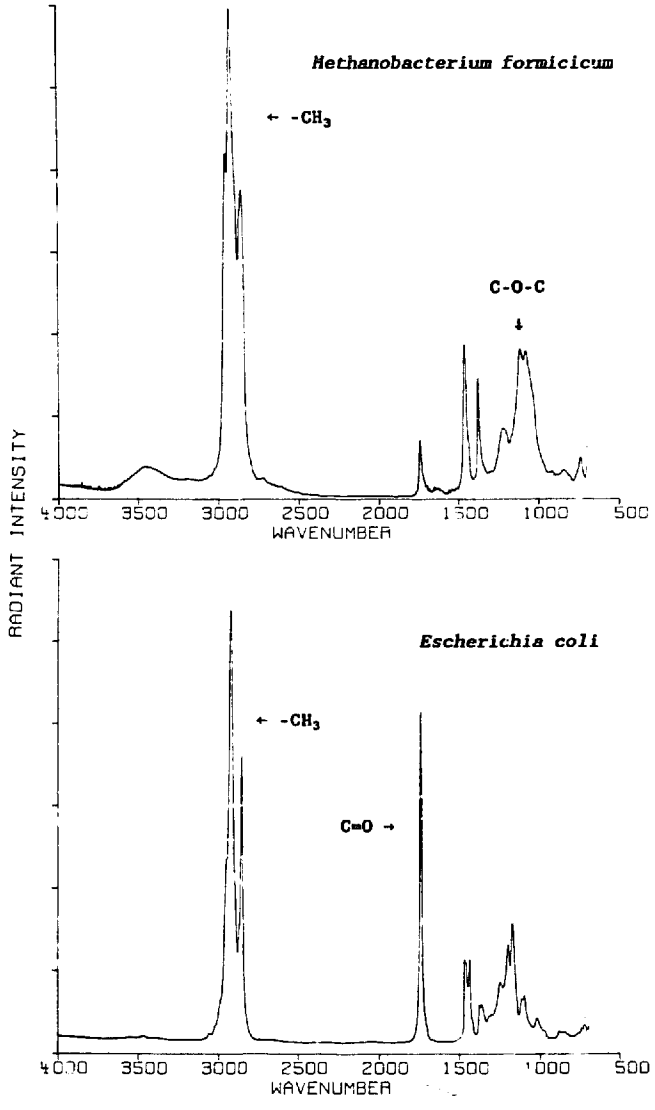

Fig. 1. Diffuse reflectance IR spectra of total lipid strong acid methanolyzates of *M. formicicum* and *E. coli*. Abbreviations of peak assignments: CH_3, methyl; C = O, ester carbonyl; C–O–C, ether.

DRIFT analysis

All DRIFT analyses [14] were performed on a Nicolet 60SX spectrometer (Nicolet, Madison, Wisconsin) equipped with Spectrotech diffuse reflectance apparatus, a globar source, a KBr beam splitter, and a narrow-band liquid N_2-cooled HgCdTe detector. Each lipid preparation was taken up in 100 μl of methylene chloride and applied to powdered KBr in a sample cup. After the solvent evaporated, the cup was placed in the N_2-purged sample compartment and 256 scans were collected and averaged. The data were collected as single-sided interferograms with a resolution of 2 cm^{-1} at a mirror retardation of 1.57 $cm \cdot s^{-1}$. Interferograms were Fourier processed using phase correction and a Happ-Genzel appodization function. The ratio between the sample and neat KBr was calculated, the baseline corrected, and the appropriate blank subtracted. The final spectra were converted to Kubelka-Munk units of radiant intensity ($W \cdot steradian^{-1}$) and smoothed using a five-point least-squares quadratic smoothing algorithm [15]. Peak assignments were made by comparison with published lipid spectra [16–19] and with those of the standard compounds. The peak height of the carbonyl stretch at 1743 cm^{-1} was expressed as a percent of the largest peak in all the spectra, the methyl peak at 2924 cm^{-1}, to correct for variation in sample size, extraction efficiency, and lipid content of the cell material.

Reliability and sensitivity of the method

The reliability of the method was tested by classifying 30 mg samples of *M. formicicum, S. acidocaldarius, T. commune, E. coli, B. subtilis*, and *M. lysodeikticus*. Only 3 mg of *T. acidophilum* was used due to limits on the amount of cell material available.

M. maripaludis and *C. welchii* were used to test the sensitivity of the method. Duplicate samples of 30, 10, 3, 1, and $\approx$ 0.5 mg were extracted and treated with the SAM procedure.

TABLE 1

HEIGHT OF DRIFT ESTER CARBONYL STRETCH PEAK (1743 ± 1.9 CM^{-1}) EXPRESSED AS PERCENT OF MAJOR METHYL PEAK (2924 ± 1.5 CM^{-1}) FOR FOUR EUBACTERIAL AND THREE ARCHAEBACTERIAL SPECIES

	Carbonyl : methyl		
	%	$\bar{x}$	SD
Archaebacteria			
M. formicicum	11.9	9.6	2.7
S. acidocaldarius	6.6		
T. acidophilum	10.3		
Eubacteria, with ester-linked lipids			
E. coli	76.6	69.5	11.3
B. subtilis	56.5		
M. lysodeikticus	75.5		
Eubacterium, with ester and ether lipids			
T. commune	28.4		

TABLE 2

SENSITIVITY OF DRIFT METHOD FOR DISTINGUISHING ARCHAEBACTERIA FROM EUBACTERIA, CARBONYL : METHYL EXPRESSED AS A PERCENT FOR DIFFERENT AMOUNTS OF LYOPHILIZED CELLS, TWO DETERMINATIONS OF EACH

mg extracted	Carbonyl : methyl		
	%	$\bar{x}$	SD
M. maripaludis, archaebacterium			
30	8.2, 9.0	7.3	2.9
10	3.3, 7.3		
3	5.1, 4.1		
1	10.6, 10.8		
0.5, 0.3	14.5, 38.7		
C. welchii, eubacterium			
30	44.9, 32.2	40.3	5.4
10	40.0, 38.9		
3	34.3, 39.4		
1	44.3, 48.3		
0.5	59.4, 75.4		

Results

DRIFT analysis

The DRIFT spectra of the strong acid methanolyzates of the total lipid of *M. formicicum* and *E. coli* are presented in Fig. 1. The locations of the ether (C–O–C, 1102 cm^{-1}), ester carbonyl (C = O, 1743 cm^{-1}), and methyl (CH_3, 2924 cm^{-1}) peaks are indicated. The heavy branching of the archaebacterial ether lipids broadened the peaks in the alkyl region between 2800 and 3000 cm^{-1}. The alkyl peaks from the eubacterial spectra were narrower and clearly separated.

Reliability of the method

The peak heights of the carbonyl stretch expressed as percent of the methyl peak for three archaebacterial and four eubacterial species are presented in Table 1. The three ester-lipid containing eubacteria were clearly separated from the ester and ether containing *T. commune*, and it from the three archaebacteria.

Sensitivity of the method

In Table 2, duplicate analyses of 30, 10, 3, 1, and <1 mg of *M. maripaludis* and *C. welchii* are compared. The reproducibility of percent carbonyl : methyl peak height for distinguishing archaebacteria from eubacteria at sample weights from 30 to 1 mg dry wt was found to be quite good. For sample weights <1 mg, the effect of background offset causes the ratio of the peak areas to increase.

Discussion

The ether adsorption peak for the archaebacterial lipids is found at $\approx$ 1102 cm^{-1}. Unfortunately, there are many peaks appearing in this region of the IR spectrum and precise assignments are difficult. However, the carbonyl moiety, characteristic of the eubacterial fatty acid methyl ester, absorbs at $\approx$ 1743 cm^{-1} where the spectra are relatively free of interfering peaks. The methyl peak at 2924 cm^{-1} was the largest peak observed in all spectra. Archaebacterial isoprenoid lipids have one out of every five carbons as methyl carbon, much more than eubacteria. The ester peak height expressed as a percent of the methyl peak was 69.5 ± 11.3 ($\bar{x} \pm$ SD) for three ester-linked lipid containing eubacteria and 9.6 ± 2.7 for archaebacteria (Table 1), clearly distinguishing the six species tested. *T. commune* is an unusual eubacterium in that it contains both ester- and ether-linked lipids [4]. It was included in the experiment since it would pose the most difficult test of the method. For this organism, the percent ester peak relative to methyl peak was intermediate between the averages for each of the two prokaryotic kingdoms, and clearly distinguished from each.

The sensitivity of the proposed method was then tested on an additional species from each kingdom: the archaebacterium *M. maripaludis* and the eubacterium *C. welchii* (Table 2). The carbonyl : methyl ratio for *M. maripaludis* was within the range of values found for the previous three species of archaebacteria tested for dry cell wt from 1 to 30 mg. The values obtained for *C. welchii* from 1 to 30 mg dry wt cells was 40.3 ± 5.4, separating it from the archaebacteria and from *T. commune.*

Conclusion

If care is taken to prevent contamination, and an appropriate blank is run in parallel to the samples and correctly subtracted from the spectra taken, 1 mg dry wt of cell material is sufficient to determine whether a pure culture is an archaebacterium or eubacterium. Using this method, 20 samples were prepared for analysis in 1 day. For comparison, the preparation of 10 samples for gas chromatographic analysis of their fatty acids requires at least 4 days. The FTIR spectrum requires 2–3 min of instrument time, while the gas chromatographic run takes 30–60 min. This method would be useful for the screening of a large number of isolates to distinguish archaebacteria from eubacteria. It would not be appropriate for mixed cultures or environmental samples.

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